Journal of Infection and Public Health 12 (2019) 49–56

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Circulation of single serotype of Dengue Virus (DENV-3) in New Delhi, India during 2016: A change in the epidemiological trend

Nazish Parveen[a], Arshi Islam[a], Ayesha Tazeen[a], Malik Hisamuddin[a,b], Mohd Abdullah[a,b], Irshad H. Naqvi[c], Md I. Faizan[a], Divya Gulyani[a], Anwar Ahmed[d,e], Shama Parveen[a,*]

[a] Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi, India
[b] School of Basic and Life Sciences, Jaipur National University, Jaipur, India
[c] Dr. M.A. Ansari Health Centre, Jamia Millia Islamia, New Delhi, India
[d] Department of Biochemistry, King Saud University, Riyadh, Saudi Arabia
[e] Centre for Excellence in Biotechnology Research, Department of Biochemistry, King Saud University, Riyadh, Saudi Arabia

ARTICLE INFO

Article history:
Received 2 November 2017
Received in revised form 20 July 2018
Accepted 27 August 2018

Keywords:
Dengue virus
Epidemiology
Phylogenetic analysis
Selection pressure
Entropy analysis

ABSTRACT

Background: Dengue is a rapidly emerging arthropod borne viral infection affecting tropical and sub-tropical regions of the world. Dengue is an acute febrile illness but sometimes causes more fatal complications like dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Delhi, the capital of India has become hyper endemic for dengue virus because all the four serotypes are circulating here.

Methods: The present study describes the identification of dengue virus from clinical samples collected from the suspected dengue patients from New Delhi, India during 2016. The CprM region of Dengue virus genome was analyzed for phylogenetic, selection pressure and Shannon entropy analyses.

Results: The present study reports circulation of a single serotype (DENV-3) in New Delhi, during 2016. The phylogenetic analysis revealed that Indian subcontinent (genotype III) of DENV-3 was circulating in Delhi during this period. Neutral selection pressure in the analyzed region revealed relatively conserved nature of this part of the Dengue virus genome. Amino acid at 31 was positively selected and had high entropy value suggesting probability of variation at this position.

Conclusions: The changing trend in circulation of dengue virus serotypes necessitates the continuous epidemiological surveillance for the dengue outbreaks in this region.

Introduction

Dengue infection is one of the most calamitous, rapidly expanding mosquito-borne viral disease that infects humans causing flu like illness (Dengue fever) but sometimes may lead to more lethal complications called Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS). The Dengue fever burden has expanded almost 30 folds in last 50 years [1]. DENV is transmitted to humans by the bite of infected *Aedes* mosquito [2]. Symptoms of dengue usually appear within 4–7 days after the bite of infected mosquito. Patients manifest with sudden high fever accompanied by severe headache, chills, fatigue, nausea, frequent vomiting, retro-orbital pain, abdominal pain, skin rash, severe joint pain, muscles pain and mild bleeding from mucosal linings which appears 2–5 days after the onset of fever.

The etiological agent of the disease is Dengue virus (DENV) which belongs to the genus *Flavivirus* and family *Flaviviradae*. The virus is single stranded and is about 11 kb in size. The genome encodes for 3 structural {capsid (C), pre-membrane (prM) and envelope (E)} and 7 non-structural proteins.

DENV exist as 4 antigenically distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) in nature. These antigenically distinct

Abbreviations: DENV, Dengue virus; SLAC, single likelihood ancestor counting; FEL, fixed effects likelihood; IFEL, internal fixed effects likelihood; REL, random effects likelihood; MEME, mixed effect model of evolution.

* Corresponding author.
E-mail addresses: shamp25@yahoo.com, sparveen2@jmi.ac.in (S. Parveen).

https://doi.org/10.1016/j.jiph.2018.08.008

serotypes are further classified into multiple genotypes. Genetic variations within the serotypes have lead to changes in virulence level and transmission of the DENV serotypes which ultimately leads to different patterns of disease severity. Infection with any of the serotypes provides lifelong protective immunity to that serotype and cross-protective immunity against the other serotypes for a short period of time probably due to antibody dependent enhancement of infection. Genotyping of the virus was done on different genomic regions in past to identify these genetic variants of dengue virus [3–6]. In this study we genetically characterized the CprM region of the dengue virus genome in patients who were tested positive for dengue fever during 2016 in New Delhi. Determination of molecular diversity of the dengue virus is essential to evaluate the impact of these genetic variants in the human population, its dispersion and virulence pattern responsible for the outbreak, as well as the origin of the new strains.

Methods

Collection of clinical samples

The study was approved by Institutional Ethics Committee of Jamia Millia Islamia and was done in accordance with the World Medical Association Declaration of Helsinki. Acute phase blood samples were drawn from the clinically suspected symptomatic patients visiting the Out Patient Department (OPD) of Dr. M. A. Ansari Health Centre of Jamia Millia Islamia, New Delhi, India. The Subject Information Sheet and Consent Form (SISCF) was obtained from patients or parents in both English and Hindi languages before the collection of samples. Demographic and clinical details of the patients were collected by the clinicians. The blood samples were transported to the Virology Laboratory within 2–3 h of collection. Serum was then separated from blood by centrifugation at 3000 rpm for 10 min at 4 °C. Aliquots of the samples were then stored at −80 °C until further use.

RNA extraction and cDNA synthesis

Viral RNA was extracted from the serum samples by using commercially available RNA Sure Virus Kit (Genetix, India) according to the manufacturer's protocol. The extracted RNA template was then reverse transcribed into cDNA using the commercially available High Capacity cDNA Reverse Transcription Kit (Applied Bio-systems, USA). The cDNA synthesis was allowed to proceed in the conditions that have been standardized in our laboratory [9].

Detection of Dengue virus by RT-PCR

All the patient's samples were tested by DENV specific RT-PCR (reverse transcriptase polymerase chain reaction) assay using published primers [7]. Dengue virus specific cDNA was amplified by two round of amplification process; external and semi nested PCR for CprM region using conditions that have been standardized in our laboratory [8]. External PCR was carried out using two consensus primers forward (D1) & reverse (D2) that anneal to any of the serotype resulting in 511 bp segment specific to all the serotypes. Amplicons of external PCR were re-amplified by semi-nested PCR using D1 as a forward primer and four other serotype specific reverse primers i.e TS1, TS2, TS3 and TS4. Semi nested PCR was initiated with the diluted amplified product (1:5 ratio) from external PCR reaction. The semi nested PCR results in amplicons of different sizes specific to each serotype (485 bp, 119 bp, 290 bp and 392 bp for DENV 1–4 respectively). Amplicons were resolved on 2% agarose gel and visualized with ethidium bromide in UV light by using a gel documentation system (Wealtec, USA).

DNA sequencing

The CprM gene segment of 290 bp of DENV-3 serotype was used for DNA sequencing. The specific bands were excised from the gel. The DNA were extracted from the gel by using QIAquick Gel Extraction Kit (Qiagen, Germany) as per manufacturer's instructions. Nucleotide sequences of CprM region of DENV-3 viruses were determined commercially in both forward and reverse directions (Applied Biosystem, USA).

Phylogenetic analysis

Identity of the obtained sequences were confirmed by BLAST tool available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Both forward and reverse sequences were manually aligned and edited to resolve the nucleotide ambiguities and to obtain consensus sequence using GeneDoc v.2.7 (http://genedoc. software.informer.com/2.7/) and BioEdit v.7.2 (http://bioedit.software.informer.com/7.2/). The study sequences were aligned with other available sequences retrieved from Genbank using CLUSTAL X2 (http://www.clustal.org/clustal2/) achieved in BioEdit v.7.2 (http://bioedit.software.informer.com/7.2/). All the new sequences of DENV-3 genome were submitted to Genbank.

The phylogenetic tree was constructed for the DENV-3 sequences by Maximum Likelihood method using algorithms implemented in MEGA6 v.6.06 software. Genetic distances were calculated using Tamura-Nei model of nucleotide substitution. The robustness of the tree was assessed with 1000 bootstrap replicates.

Selection pressure analysis

Selection pressure was estimated for the individual codons in the CprM region of the sequences using a Datamonkey web-server (http://www.datamonkey.org/). Strength of selection pressure relies on dN/dS (ratio of non-synonymous to synonymous mutations). Calculation of dN/dS was done using five different approaches, including SLAC, FEL, IFEL, REL and MEME under the F81, HKY85 and REV models of nucleotide substitution. Both average dN/dS for aligned sequences as well as dN/dS per codon site were calculated. The positively selected sites are the sites for positive selection defined under two different significant values; P-value (0.05–0.25) for SLAC, FEL, IFEL and MEME and Bayes factor (25–125) for REL.

Shannon entropy analysis

BioEdit v.7.2 software was used for the Shannon entropy analysis. It is used for the identification of possible variability/mutability at particular codon position. The values obtained from the software through various entropy calculations were then imported in Microsoft Excel to plot the entropy graph.

Results

Patient's clinical features

Symptomatic patients attending the Out Patient Department (OPD) of Dr. M. A. Ansari Health Centre of Jamia Millia Islamia were recruited in the study over a period of 3 months from September to November, 2016 in Delhi. A total of 71 acute phase blood samples were collected from the symptomatic patients during the investigation. Patients manifested the classical symptoms of fever accompanied by history of any of the following symptoms; headache, rashes, vomiting, nausea, body ache and weakness were enrolled in the study. Fever was the most common symptom that was observed in all the patients. Duration of fever ranged from 1

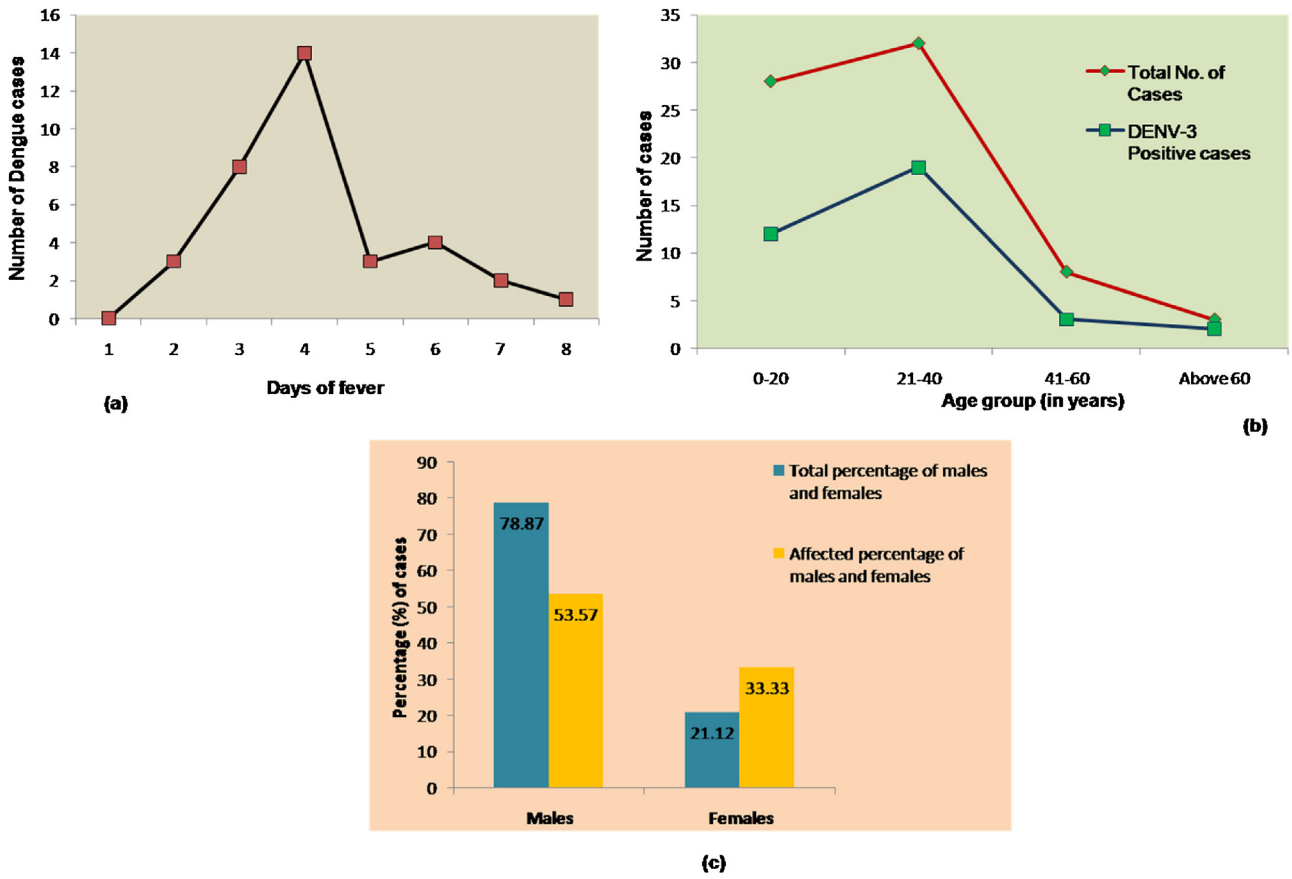

Fig. 1. Demographical details of the patients. (a) Graph showing correlation between number of DENV-3 positive cases and fever days. (b) Age wise distribution of total number of cases and dengue positive cases. (c) Gender wise distribution of the dengue cases.

to 10 days. Supplementary Table 1 shows the demographical and clinical details of the patients enrolled in the study.

Screening of clinical samples by RT-PCR

All the 71 samples collected during the study were tested for Dengue virus infection by RT-PCR. Thirty five samples (49.29%) were found to be positive for DENV out of the 71 samples. Only DENV-3 serotype was identified in all the positive samples.

Demographical analysis of patients having Dengue viral infection

Occurrence of dengue viral infection was correlated with the demographics (age & sex) of the patients. Fifty six patients (78.87%) were males and 15 (21.12%) were females with a male/female ratio of 4:1. More males were affected (53.57%) as compared to females (33.33%) with affected male/female ratio of 1.6:1. The mean age of Dengue positive patients was 26.18 years (SD ± 13.14 years) and the average duration of Dengue fever was 4 days (SD ± 1.97 days) (Fig. 1).

The age of the patients ranged from 3 to 70 years. The patients were grouped into 4 based on their age: 0–20, 21–40, 41–60 and above 60 years. The age group analysis of the patients revealed that the maximum numbers of Dengue positive cases (19) were found in the age group of 21–40 years followed by 0–20 years (12) which accounted for 52.7% and 33.3% of the positive cases respectively (Fig. 1).

DNA sequence analysis

The CprM region of the DENV-3 genome was sequenced for 8 positive samples. The samples were sequenced in both forward and reverse direction. All the obtained DENV-3 sequences were confirmed by BLAST. These sequences were submitted in Genbank database with Accession numbers of MF038783 to MF038788, KY099619 and KY099620.

Phylogenetic analysis

Phylogenetic analysis of DENV-3 serotype was carried out for the present investigation. The H87 strain of genotype V of DENV-3 (GenBank Accession number M93130) was used as the prototype strain. The aligned region was 240 bp that corresponds to 131 to 370 bp of the CprM region offull genome the prototype strain. Eighty sequences (8 studied and 72 other sequences of different genotypes of DENV-3 (GenBank) were aligned to construct Maximum Likelihood tree. Phylogenetic analysis clustered all the studied sequences with the genotype III (Indian subcontinent) (Fig. 2). The study sequences showed nucleotide and amino acid distances of 8.5% to 9.5% and 2.5% to 5.1% respectively with respect to the prototype strain. Further, nucleotide and amino acid distances of upto 1.7% and 2.5% respectively have been shown among themselves.

A total of 24 mutations were observed at nucleotide level in all the strains with respect to the prototype strain. Four amino acid mutations were identified at different sites in all the study sequences. All the strains showed two common mutation i.e; Val15Ala and Arg31Lys. Additionally Ile9Phe mutation was shown

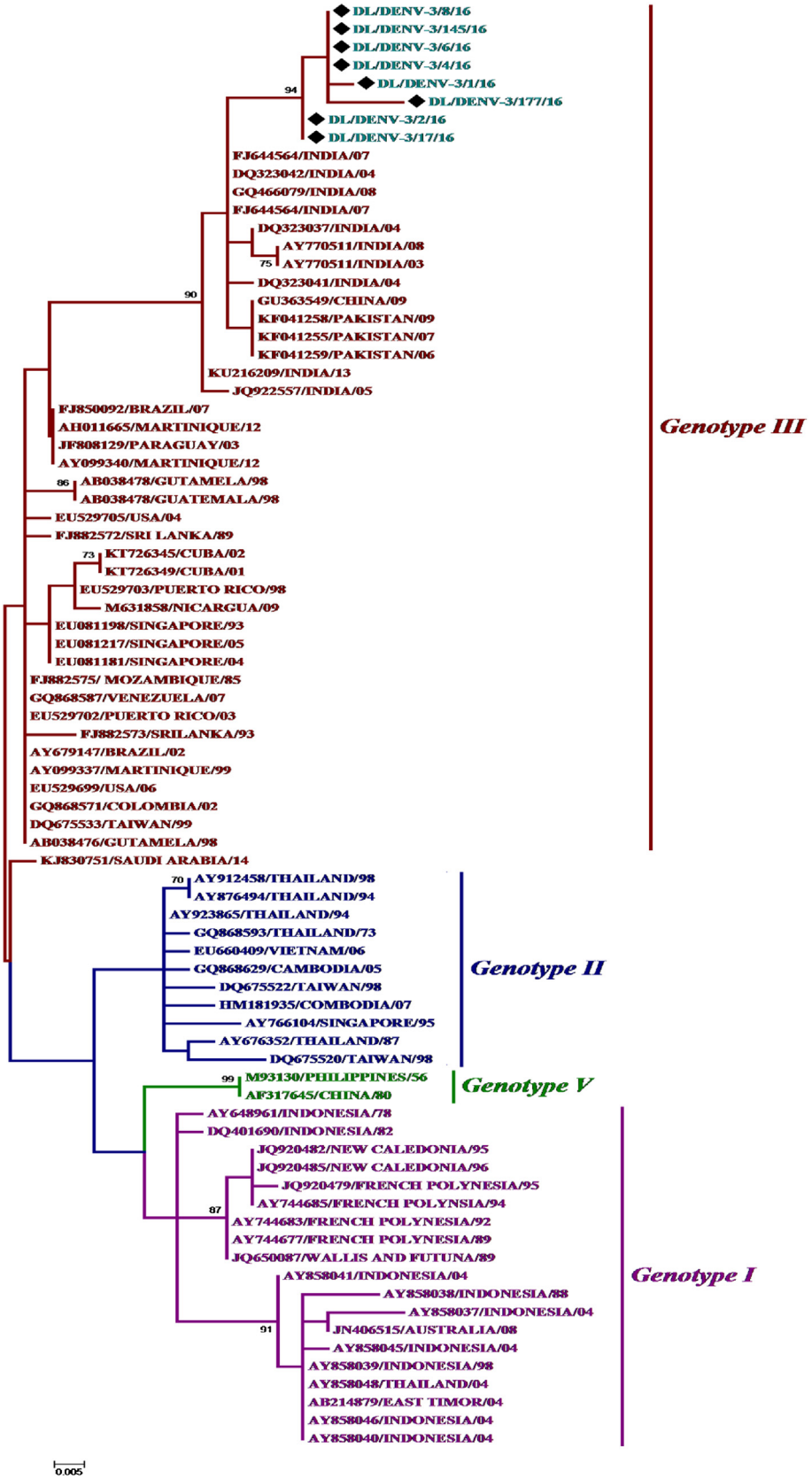

Fig. 2. Maximum likelihood phylogenetic tree of DENV-3 strains. The study sequences are marked by the symbol ♦. Bootstrap values are represented by the number on nodes generated by 1000 replications. The strains used to construct the tree are represented by the strain name, followed by country/region and year of isolation/submission of strain. The study sequences clustered with Genotype III (Indian subcontinent).

Fig. 3. Representation of mutations (amino-acid substitutions) of DENV-3 strains in the CprM region with respect to the prototype strain (H87). The sequence corresponds to the 9–88 amino acids of the prototype strain. Changes at amino acid positions in the studied strains are shown by arrows.

by all the strains except DL/DENV-3/177/16 and DL/DENV-3/2/16. Strain DL/DENV-3/177/16 showed an additional mutation i.e; Ser71Val. Fig. 3 summarizes all the mutations at different positions as compared to the prototype strain.

Selection pressure analysis

The selection pressure analysis was done for the CprM region of all the sequences including prototype and study strains to determine the residues under positive selection for mutation. The results are summarized in Table 1 which shows dN/dS values, p-values, bayes factor for codon positions under positive selection using different methods. The mean dN/dS by SLAC method showed a low ratio ranging from 0.07 to 0.11 suggesting that codons were under purifying selection. SLAC analysis showed no positive selection sites. The codon positions 9, 15, 23, 31, 71 and 78 were found to be under positive selection by different methods. Amino acid at position 31 was found to be positively selected by all the four methods (IFEL, FEL, REL, MEME) under all nucleotide substitution models with significant values (p-value = 0.18 and Bayes factor = 93.69).

Shannon entropy analysis

The Shannon entropy analysis of CprM region for DENV-3 genome was done to analyze the variable sites among the sequences. Different position of codons and their respective entropy scores are summarized in Shannon entropy (H) plot to determine the variability/mutability of the amino acids (Fig. 4). A value of 0.2 entropy score was set as the threshold value in the selection process of variable sites. A total of 5 variable sites were identified at codon positions 9, 15, 31, 61 and 78. Two of these amino acid at position 31 and 78 were found to have higher values of entropy 0.67 and 0.54 respectively.

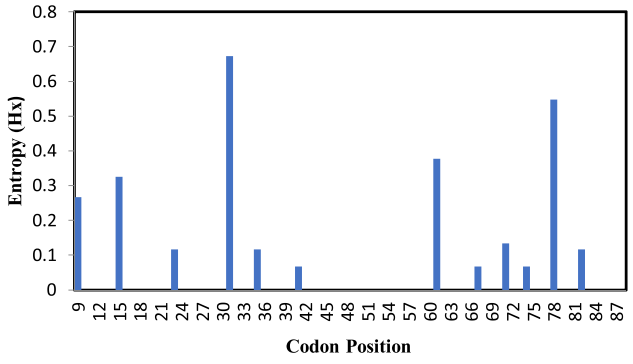

Fig. 4. Shannon entropy plot of CprM region of the DENV-3 strains. Maximum entropy value reflects to the greater chance of variability/mutation at that position in the protein sequence.

Discussion

Dengue fever is emerging as one of the most devastating health problem. In last decades Delhi, the capital of India has become hyper-endemic for Dengue virus because all the four serotypes have been implicated in various outbreaks at different times. Several epidemics of dengue fever have been reported in Delhi in various investigations [5,8–18]. However, concurrent infection with multiple serotypes has also been reported in previous investigations [19–21]. Enhanced transmission of the virus to different geographical regions is probably due to climatic changes (high humidity, high temperature and heavy rainfall) and inadequate water drainage system which leads to the rapid proliferation of mosquitoes [22]. In addition, dense human population, rapid urbanization and increasing globalization also contribute towards frequent outbreaks of dengue fever in different regions. Therefore, regular surveillance of the Dengue virus is needed especially in the endemic regions to provide insight into the epidemiology and evolutionary pattern of this emerging viral pathogen.

The genotyping based on the CprM gene is comparatively easier and more economical alternative due to utilization of a single set of primer pair for both amplification and sequencing. In addition, phylogenetic trees based on CprM and E gene regions are found to have a similar topology with no discrepancy between genotypes. Therefore our study was planned to examine the molecular characterization of dengue virus using CprM region circulating in the city of New Delhi, India during 2016. Dengue virus was detected in 49% of the samples by RT-PCR in the present study. Former studies from our laboratory have identified DENV in 49% of the sample during 2011 [9], 71% in 2013 [19], 69% during four year study period from 2011 to 2014 [22], 80% in 2014 [17] and 29% in the year 2015 [16]. Similar to our observations Kerala and Madhya Pradesh from India also identified DENV in 49% of the clinically suspected patients [6,23].

A change in the circulation pattern of Dengue serotypes has been reported from Delhi in terms of prevalent serotype. The DENV-2 serotype dominated the Dengue fever during 2003 to 2006 [5,7,24–26]. DENV-3 emerged as a dominant serotype in the year 2003 which continued in circulation till 2006 [5,27]. In addition, 2006 also reported the emergence of DENV-1 in 30% of the cases [5]. Subsequently, the predominance of DENV-1 was reported till 2010 [12,14,28]. An earlier study from our laboratory reported that the DENV-1 and DENV-2 serotypes co-circulated in almost equal proportions in 2011 [8]. A switch in the prevailing serotype occurred again in 2012 due to co-dominant circulation of DENV-2 and DENV-3 [29]. DENV-2 dominated during 2012, 2013 and 2015 [8,15]. Subsequently, DENV-1 dominated in 2014, reporting 70% of the cases [16]. Further, during 2016 a change in the circulating serotype occurred leading to dominance of DENV-3 in this region.

The striking features of our study was the identification of only a single serotype, DENV-3 from Delhi during 2016. No other serotype was detected during the study suggesting the deviation in circulation from DENV-1 and DENV-2 towards the predomi-

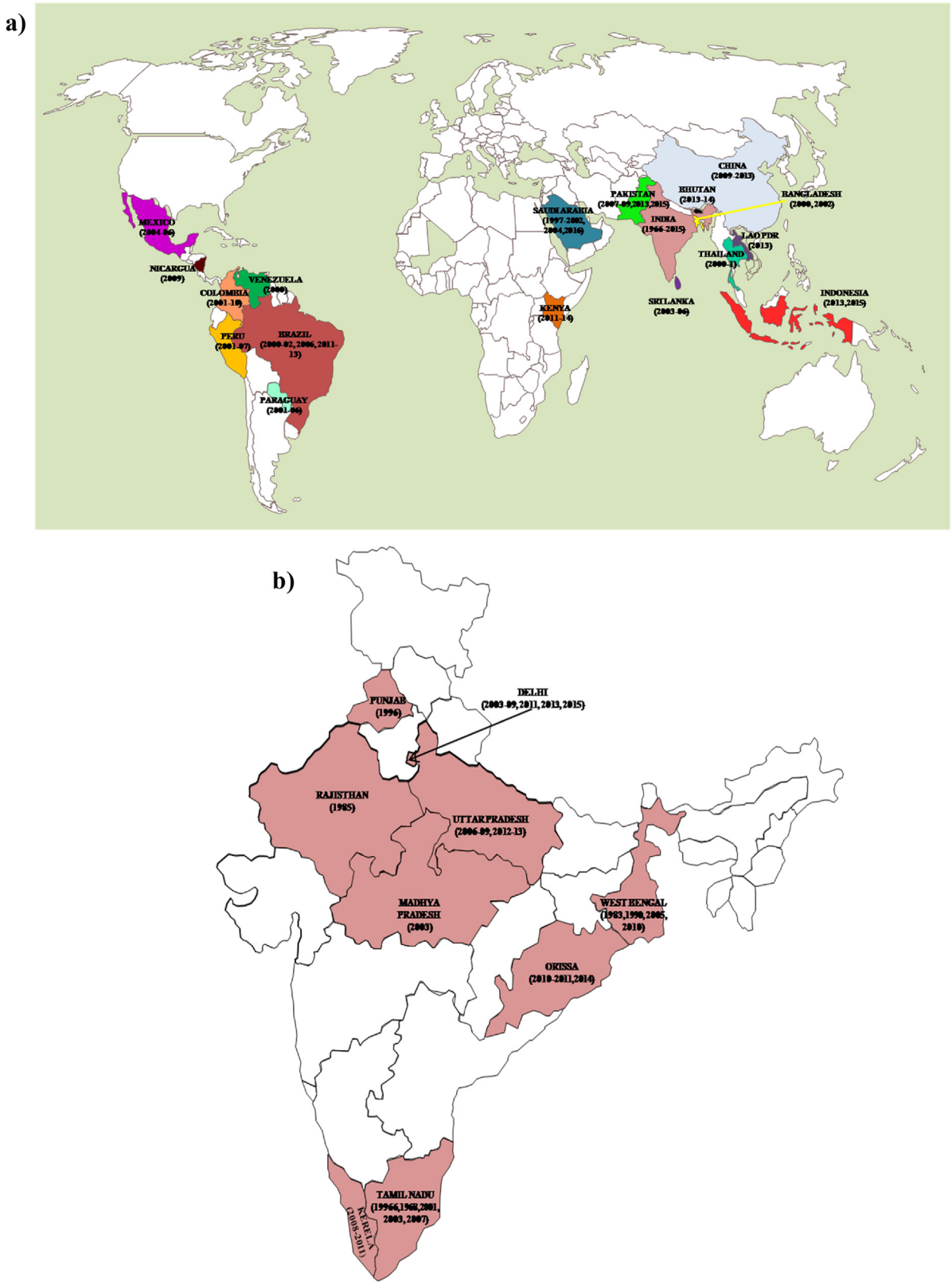

Fig. 5. (a) World map showing DENV-3 infections in different geographical regions. Shaded regions showing infected countries along with year of detection of infection. The freely available world map was downloaded from the presentationmagzine.com website (https://www.presentationmagzine.com/world-maps-vector-editable-507.htm).The map was edited in Microsoft power point. (b) Map of India showing DENV-3 infections in different geographical regions. Shaded regions showing infected states along with year of detection of infection. The freely available map of India was downloaded from the presentationmagzine.com website (https://www.presentationmagazine.com/powerpoint-map-of-india-647.htm). The map was edited in microsoft power point.

Table 1
Selection pressure analysis of CprM region of DENV-3 genome using IFEL, FEL, MEME and REL methods.

IFEL (Internal Fixed Effects Likelihood)							FEL(Fixed Effects Likelihood)				MEME (Mixed Effect Model of Evolution)			REL (Random Effects Likelihood)				
Nucleotide substitution method	F81		HYK85		REV		HYK85		REV		F81	HYK85	REV	F81		HYK85		REV
Codon	p-value	dN/dS	p-value	dN/dS	p-value	dN/dS	p-value	dN/dS	p-value	dN/dS	p-value	p-value	p-value	B-Factor	dN/dS	B-Factor	dN/dS	B-Factor	dN/dS
9	–	–	–	–	0.2461	3.4567	–	–	–	–	0.0898	–	–	–	–	–	–	–	–
15	0.218	3.131	0.2132	3.3617	–	–	–	–	–	–		–	–	–	–	–	–	–	–
23	–	–	–	–	–	–	–	–	–	–	0.1898	0.1898	0.1898	–	–	–	–	–	–
31	–	–	0.1687	4.2558	0.1838	6.2482	0.148	1.272	0.1652	5.428	0.1735	0.1735	0.1735	30.042	0.01625	93.6941	0.07875	–	–
71	–	–	–	–	–	–	–	–	–	–	0.0008	0.0008	0.0008	–	–	–	–	–	–
78	–	–	0.1707	4.2382	0.9003	6.0916	–	–	–	–	–	–	–	–	–	–	–	–	–

dN/dS — ratio of non-synonymous to synonymous mutation.
B-Factor — Bayes factor.

nant circulation of DENV-3 in this population. Similarly, study from Pakistan has also reported the detection of only DENV-3 in all the positive samples during 2015 [30]. DENV-3 and DENV-2 were reported to be more likely cause of DHF during secondary infections than other serotypes as reported earlier [31]. Studied strains of DENV-3 were clustered in Genotype III (Indian subcontinent) by phylogenetic analysis. The analysis also concluded that the DENV-3 study sequences grouped with the strains from China (unpublished), Pakistan [32] and India [32–34]. Circulation of Indian subcontinent genotype in India was also reported in some recent investigations [15,21,30]. DENV-3 serotype has been reported from different geographical regions including India (Fig. 5). The references for these countries are available on request.

Dengue fever affects all the age groups but certain age groups may be more prone to the infection. Patients in the age group of 21–40 years were more affected by Dengue fever in the present investigation. Similarly previous studies from Delhi also showed maximum DENV infection in this age group [10,17,35,38]. However, various studies from South India reported maximum cases of Dengue fever in the lower age group (0–18 years) than adults [36]. Statistical correlation of the demographic details of the patients with Dengue positive cases did not reveal any striking differences (data not shown). Additionally, it was also observed that more number of males was affected as compared to females that coincided with the fact that more number of male patients were recruited in the study. Additional investigations on larger groups of patients together with detailed statistical analysis of the data will provide the detailed information on DENV affected individuals.

DENV proteins adapts to the environmental changes and to different hosts immune system (human and mosquito) by selection of synonymous mutations. Changes in environmental conditions have led to shift the selective pressures on genomic regions associated with adaptation of the virus and virulence. The capsid pre-membrane region is part of the genome involved in the formation of basic structure of the virus. Therefore, mutations in this region can also lead to structural and functional changes. Therefore the selection pressure analysis of the CprM region was done to investigate the non-synonymous to synonymous mutation ratio and the sites under positive selection pressure were identified. The low ratio of mean dN/dS suggested evidence of purifying selection in the CprM region of Dengue virus. In the present study, the positively selected sites were determined for the studied dataset suggesting stochastic process of evolution. Likewise, purifying selection in the CprM region of DENV-3 has also been reported previously [37,38]. Six different codon positions at 9, 15, 23, 31, 71 and 78 of CprM region of DENV-3 was identified to be under positive selection. The codon position 31 was positively selected by all the nucleotide substitution models using different methods and also had highest entropy value suggesting probability of variation. These predicted mutations might lead to changes in the virus structure thereby affecting the structure-function relationship. Additional detailed analysis of these mutations by site directed mutagenesis will provide insight into the role of these mutations in the life cycle of the virus.

Conclusions

Present study thus reports the circulation of only single serotype (DENV-3) from New Delhi during 2016. The molecular characterization of the Dengue virus strains revealed that Indian Sub-continent genotype was circulating in Delhi during this period. The immediate shift in circulation and dominance pattern of DENV-3 replacing the earlier circulating serotypes (DENV-1 and DENV-2) during 2016 has shown a significant change in the Dengue epidemiology. This serotypic shift is likely to pose a threat to the secondary infections

with DENV due to antibody dependent enhancement of disease. Neutral selection pressure in this genotype suggested a lower rate of mutations in this region of Dengue virus genome. Additionally, detailed evolutionary analysis using Bayesian methods can be used for determining evolutionary trajectory of this emerging viral pathogen in Indian settings. Further, correlation of the disease severity with the individual serotypes is another aspect that can be perused in future investigations. These investigations will assist in effective control and management of the epidemics.

Funding

No funding Sources.

Competing interests

None declared.

Ethical approval

The study was approved by Institutional Ethics Committee, Jamia Millia Islamia.

Acknowledgement

Nazish Parveen was supported by research fellowship of the University Grant Commission, Government of India for the present study. The study was also supported by Kind Saud University, Deanship of Scientific Research, College of science Research Centre.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jiph.2018.08.008.

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